

# Suppressing AP1 Factor Signaling in the Suprabasal Epidermis Produces a Keratoderma Phenotype

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Keratodermas comprise a heterogeneous group of highly debilitating and painful disorders characterized by thickening of the skin with marked hyperkeratosis. Some of these diseases are caused by genetic mutation, whereas other forms are acquired in response to environmental factors. Our understanding of signaling changes that underlie these diseases is limited. In the present study, we describe a keratoderma phenotype in mice in response to suprabasal epidermis-specific inhibition of activator protein 1 transcription factor signaling. These mice develop a severe phenotype characterized by hyperplasia, hyperkeratosis, parakeratosis, and impaired epidermal barrier function. The skin is scaled, constricting bands encircle the tail and digits, the footpads are thickened and scaled, and loricrin staining is markedly reduced in the cornified layers and increased in the nucleus. Features of this phenotype, including nuclear loricrin localization and pseudoainhum (autoamputation), are characteristic of the Vohwinkel syndrome. We confirm that the phenotype develops in a loricrin-null genetic background, indicating that suppressed suprabasal AP1 factor function is sufficient to drive this disease. We also show that the phenotype regresses when suprabasal AP1 factor signaling is restored. Our findings suggest that suppression of AP1 factor signaling in the suprabasal epidermis is a key event in the pathogenesis of keratoderma.

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## INTRODUCTION

Keratodermas comprise a group of disorders characterized by thickening of the epidermis with marked hyperplasia and hyperkeratosis (Christiano, 1997). Keratodermas include both inherited and acquired forms. Inherited forms of this disease are associated with expression of mutant intermediate filaments (keratins), cornified envelope-associated proteins (loricrin, transglutaminase), cohesion proteins (desmoglein 1, plakophilin, desmoplakin), proteins of cell-to-cell communication (connexins), and transmembrane proteins (cathepsin C) (Christiano, 1997; Kimyai-Asadi *et al.*, 2002; Amagai and Stanley, 2012). Most of these mutations are autosomal dominant, although some are recessive (Kimyai-Asadi *et al.*,

2002). In addition, some forms of keratoderma are acquired in response to treatment with drugs, exposure to chemicals, malnutrition, infection, or response to cancer (Patel *et al.*, 2007). The underlying biochemical changes in these diseases are not well understood.

The Vohwinkel syndrome is a form of keratoderma characterized by honeycomb-like palmoplantar keratoderma, hyperkeratosis, prominent tissue buildup on the dorsal hands and feet, pseudoainhum with digit amputation, and hearing impairment (OMIM 124500) that is caused by a mutation in the connexin 26 gene (Maestrini *et al.*, 1999). In addition, Camisa and Rossana reported a variant of the Vohwinkel syndrome characterized by ichthyosiform dermatosis and no hearing impairment (OMIM 604117) (Camisa and Rossana, 1984; Schmuth *et al.*, 2004). This autosomal-dominant disease, also called loricrin keratoderma or keratoderma with ichthyosis, is caused by mutation of the loricrin gene (Korge *et al.*, 1997). The loricrin mutations are frameshift changes due to a single nucleotide insertion in the loricrin-coding sequence. The most frequent mutation is 730insG (Korge *et al.*, 1997; Maestrini *et al.*, 1996; Takahashi *et al.*, 1999; Matsumoto *et al.*, 2001; O'Driscoll *et al.*, 2002; Gedicke *et al.*, 2006; Drera *et al.*, 2008), although mutations are also observed at 662insT (Armstrong *et al.*, 1998), 709insC (Ishida-Yamamoto *et al.*, 1997), and c.545-546insG (Song *et al.*, 2008). These mutations result in synthesis of an extended form of loricrin that includes a nuclear localization signal. Instead of being incorporated into the cornified envelope, the mutant loricrin translocates into the nucleus

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Abbreviation: rTA, reverse tetracycline activator

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where it produces changes that are not well characterized (Ishida-Yamamoto *et al.*, 1998b, 1999, 2000).

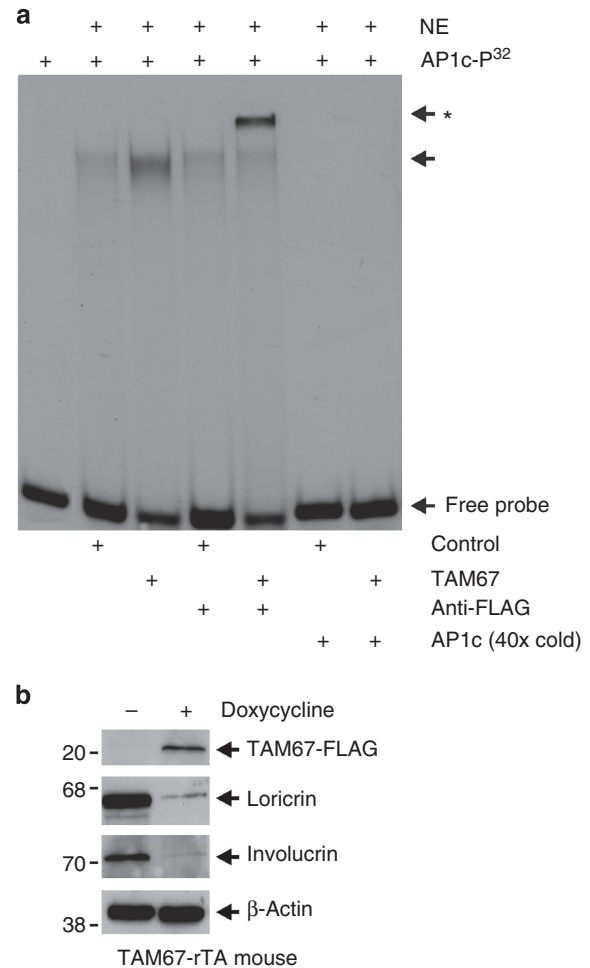
Mouse model studies support a role for mutant loricrin in the pathogenesis of loricrin keratoderma. Overexpression of wild-type human loricrin in mouse epidermis shows a normal phenotype that is associated with incorporation of human loricrin into the murine cornified envelope (Yoneda and Steinert, 1993). Loricrin knockout mice have also been produced, and these mice display only a mild phenotype (Koch *et al.*, 2000; Jarnik *et al.*, 2002). In contrast, transgenic mice expressing mutant loricrin display features of loricrin keratoderma (Suga *et al.*, 2000), and this phenotype is more dramatic when the mutant is expressed in the absence of wild-type loricrin (Suga *et al.*, 2000). The phenotype includes nuclear localization of loricrin, hyperproliferation, hyperkeratosis, parakeratosis, and pseudoainhum of the tail (Suga *et al.*, 2000). These findings argue that this is a gain-of-function mutation and that wild-type loricrin is not necessary for the phenotype. However, beyond the accumulation of nuclear loricrin, subsequent events in disease progression are not understood. Recent studies, using HaCaT cell cultures expressing mutant loricrin, suggest that nuclear accumulation of loricrin alters intracellular signaling and that this may contribute to the keratoderma phenotype (Yoneda *et al.*, 2010a, b). These findings suggest that the accumulation of mutant nuclear loricrin may influence a host of cell processes that lead to enhanced cell proliferation.

In the present study, we describe mice that express tetracycline-inducible dominant-negative c-jun (TAM67) in the suprabasal murine epidermis. Dominant-negative c-jun dimerizes with all jun/fos factors to inhibit transcription (Brown *et al.*, 1994). Jun/fos factors are key controllers of keratinocyte proliferation and differentiation (Efimova *et al.*, 1998; Efimova and Eckert, 2000; Efimova *et al.*, 2002, 2003; Eckert *et al.*, 2004). Remarkably, these mice develop an authentic loricrin keratoderma-like phenotype. Moreover, studies in loricrin-null background suggest that loricrin is not required for this response. On the basis of these findings, we suggest that reduced suprabasal AP1 factor signaling in the suprabasal epidermis is sufficient to cause keratoderma, and that suppressed suprabasal epidermal AP1 factor function may be involved in a range of keratoderma types.

## RESULTS

### TAM67 in suprabasal epidermis

SKH1-TAM67-rTA mice are engineered to encode the TetO-TAM67-FLAG and hINV-rTA gene expression cassettes. The hINV-rTA construct uses the human involucrin promoter to target expression of the rTA to the suprabasal epidermis. Doxycycline binding produces an active form of rTA, which binds to TetO to drive suprabasal expression of TAM67-FLAG. TAM67 forms inactive heterodimers with other AP1 factors, which interact with AP1 response elements in a normal manner but do not activate transcription. This results in reduced expression of AP1-responsive genes (Han *et al.*, 2012). As an initial step in characterization of the role of TAM67-FLAG in the epidermis, we confirmed that TAM67-FLAG interacts with AP1 response elements and reduces



**Figure 1. TAM67-FLAG interacts with AP1 response element and suppresses AP1-responsive genes.** SKH1-TAM67-rTA mice were treated with (+) or without (–) 2 mg ml<sup>–1</sup> doxycycline in drinking water for 3 days.

(a) Interaction of TAM67 with AP1 site consensus element. Epidermis from control and TAM67-expressing epidermis were collected by high-temperature separation (Rorke *et al.*, 2010), and nuclear extracts were prepared and incubated with AP1c-P<sup>32</sup> as indicated. NE, nuclear extract. The arrow indicates migration of the mobility shifted bands, and the arrow/asterisk indicates migration of the supershifted band (anti-FLAG-treated sample). A 40-fold excess of AP1c was added as indicated to demonstrate binding specificity. (b) Murine epidermis was collected free of the dermis by high-temperature separation as previously described (Rorke *et al.*, 2010). Total extract was prepared for immunoblot to detect the indicated proteins. TAM67-FLAG was detected with anti-FLAG. Similar results were observed in each of the three experiments.

expression of AP1 target genes. TAM67-rTA mice were treated for 3 days with or without doxycycline, and nuclear epidermal extracts were prepared. Figure 1a shows an increase in the quantity of shifted AP1c-P<sup>32</sup> probe in extract prepared from TAM67-expressing epidermis, and that this binding is reduced by addition of excess radioinert AP1c. In addition, direct TAM67-FLAG binding to AP1c-P<sup>32</sup> was confirmed by anti-FLAG supershift (Figure 1a). Expression of TAM67 in this tissue is expected to reduce expression of AP1 factor-regulated genes. To assess this, we monitored the

expression of involucrin and loricrin, two genes that require AP1 transcription factors for expression (Yoneda *et al.*, 1992; Jang and Steinert, 2002; Eckert *et al.*, 2004). TAM67-rTA mice were treated with or without 2 mg ml<sup>-1</sup> doxycycline for 3 days, and total epidermal extracts were assayed for involucrin and loricrin. We find that both involucrin and loricrin levels are reduced in TAM67-expressing epidermis (Figure 1b), thereby providing biochemical evidence for a TAM67 impact on the tissue.

#### The phenotype of SKH1-TAM67-rTA mice

To characterize the phenotype of SKH1-TAM67-rTA mice, littermates were treated with or without 2 mg ml<sup>-1</sup> doxycycline in drinking water for 0–21 days. The SKH1 genetic background was used, as the hairless phenotype permits enhanced visualization of the phenotype. However, it is important to note that the mice are completely immune competent. Figure 2a shows that TAM67-FLAG expression is associated with the development of a honeycomb epidermal phenotype that is evident during the first week and that this phenotype progresses such that the mice develop extensive hyperkeratosis by 21 days. Figure 2b shows a closer view of the 21 day doxycycline-treated mouse, illustrating the extensive hyperkeratosis and fissures that develop at the back of the animal, and Figure 2c shows the hyperkeratosis that develops at the bottom of the feet and the unusual “club” appearance of the nails. The toes also display a tendency toward pseudoainhum at the junction between the foot and digits. We next examined epidermal histology. Figure 2d shows the thin epidermis and normal keratinization characteristic of control mice, whereas Figure 2e shows the extensive hyperkeratosis observed in the epidermis of doxycycline-treated mice. Figure 2f shows the extensive parakeratosis (nuclei retention, arrows) in dead cells of the cornified layer.

As mutant loricrin redistributes from the cell periphery to the nucleus in human keratoderma, we examined the tissue and subcellular distribution of murine loricrin in the TAM67-expressing mice (Ishida-Yamamoto *et al.*, 1998a; Ishida-Yamamoto, 2003). Figure 2g shows a thin margin of loricrin staining in the epidermal cornified layer of control mice. Much to our surprise wild-type loricrin redistributes to the nucleus in the suprabasal epidermis of TAM67-FLAG-expressing mice (Figure 2h). Figure 2i shows sections co-stained with anti-loricrin and 4' 6-diamidino-2-phenylindole, which confirms nuclear loricrin localization in suprabasal cells. Figure 2j shows that doxycycline treatment results in expression of TAM67-FLAG and confirms the complete lack of expression in the absence of doxycycline. These studies show that SKH1 mice expressing TAM67 in the suprabasal epidermis develop a phenotype that includes progressive epidermal hyperkeratosis, which is associated with parakeratosis, hyperkeratosis of the skin and base of the feet, and nuclear accumulation of loricrin. This phenotype closely resembles features of human keratoderma (Camisa and Rossana, 1984; Schmuth *et al.*, 2004).

#### Rapid onset of phenotype

We next studied the time course of phenotype onset in order to understand the sequence of events during disease

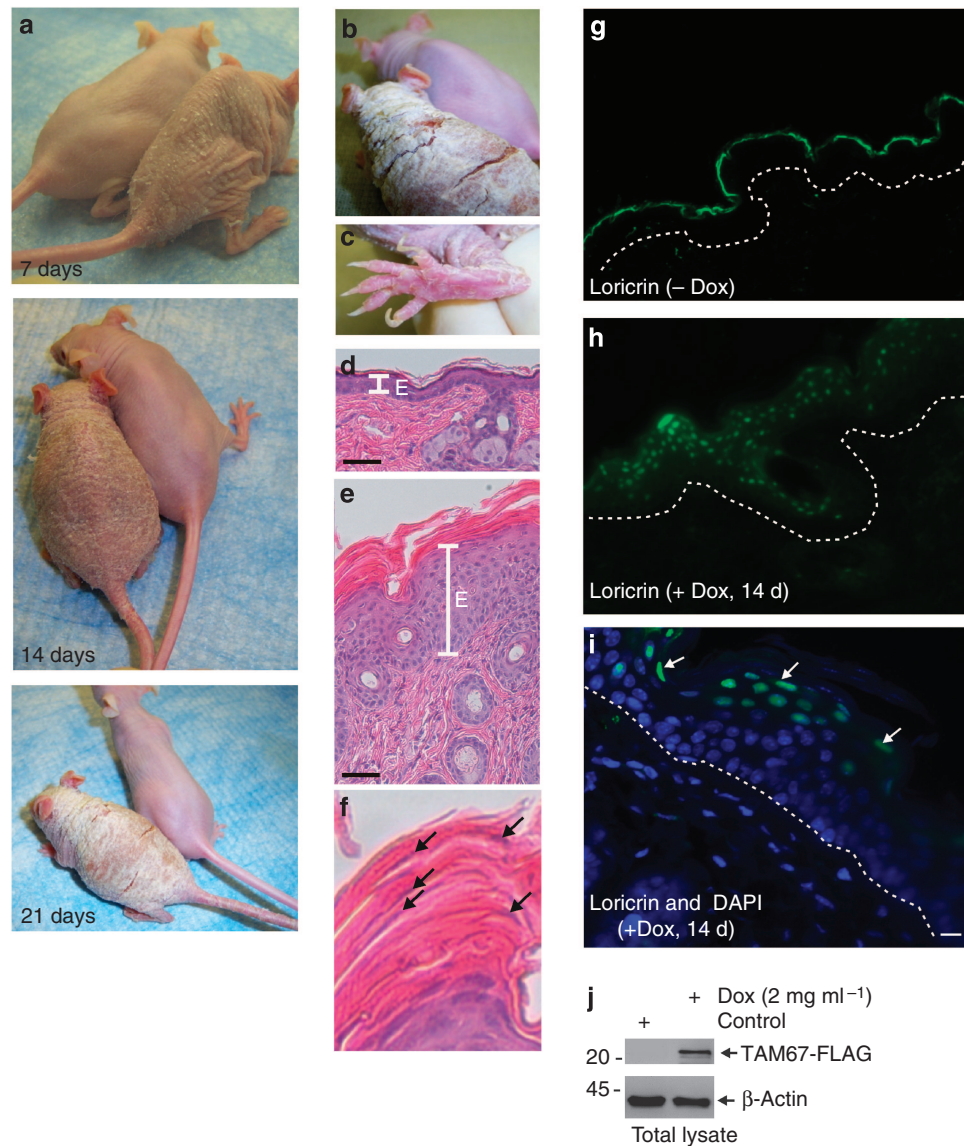
development. SKH1-TAM67-rTA mice were treated with doxycycline, and the epidermis was harvested at 0, 12, 24, 48, and 72 hours after initiation of treatment. Figure 3a shows initial evidence of nuclear TAM67-FLAG accumulation in epidermal suprabasal cells at 12 hours with continued accumulation at subsequent times (arrows). TAM67-FLAG level, as measured by immunoblot, is maximal by 48 hours and is maintained thereafter (not shown). Figure 3a shows that no TAM67-FLAG is detected in the nuclei of basal cells. To determine the time of initiation of epidermal cell hyperproliferation, we monitored the Ki67 level. Figure 3b shows increased Ki67 staining that is evident at 48 and 72 hours and is associated with increased epidermal thickness as detected in hematoxylin and eosin sections (Figure 3a). We also monitored loricrin subcellular distribution during this time course (Figure 3b). At time zero, loricrin is present as a thin band in the cornified layer, but at 24, 48, and 72 hours after activation of TAM67-FLAG expression loricrin assumes a punctate intracellular localization (Figure 3b).

We examined loricrin and TAM67-FLAG localization in detail by co-staining sections with anti-loricrin (red) and anti-TAM67-FLAG (green). At time zero, loricrin is detected as part of the cornified envelope (red) and, as expected, no TAM67-FLAG (green) is detected (Figure 4). At 12 hours after initiation of doxycycline treatment, TAM67-FLAG is detected in the nuclei, whereas loricrin remains associated with the cornified envelope. However, by 24 hours there is clear evidence for redistribution of loricrin into some nuclei in the suprabasal layer (red). Moreover, this accumulation is maintained at 48 and 72 hours (Figure 4) and at 14 days (Figure 2h and i). These findings indicate that nuclear loricrin accumulation is an early event in phenotype development and that nuclear accumulation is sustained thereafter. It is interesting to note that loricrin is present in nuclei that are strongly and weakly TAM67-FLAG positive (Figure 4), suggesting that TAM67-FLAG does not directly act to move loricrin to the nucleus. We speculate that loricrin may be moved to the nucleus via carrier protein-dependent transport, but this remains to be determined.

#### Pseudoainhum

Pseudoainhum (constriction) and autoamputation of the digits is a signature phenotype of loricrin keratoderma patients and is also observed in mutant loricrin-expressing mice (Ishida-Yamamoto *et al.*, 1997; Suga *et al.*, 2000). As shown in Figure 5, TAM67-expressing mice develop a severe pseudoainhum response. Constriction bands accumulate on the tail and are particularly evident after removal of doxycycline when the phenotype is permitted to normalize. The mouse in Figure 5 was treated with doxycycline for 21 days followed by removal of doxycycline. The photographs (Figure 5a and b) were taken at 7 and 11 days after doxycycline removal. This shows that the accumulated scale is lost with the loss of TAM67 expression, indicating that the phenotype is reversible (Figure 5a) with some residual scarring that slowly normalizes (Figure 5b). Figure 5c and d shows the tails of two mice at 11 days after removal of doxycycline treatment. The arrows indicate some of the restriction bands on the tail. A significant number of mice lose all or a portion of the tail. Figure 5e





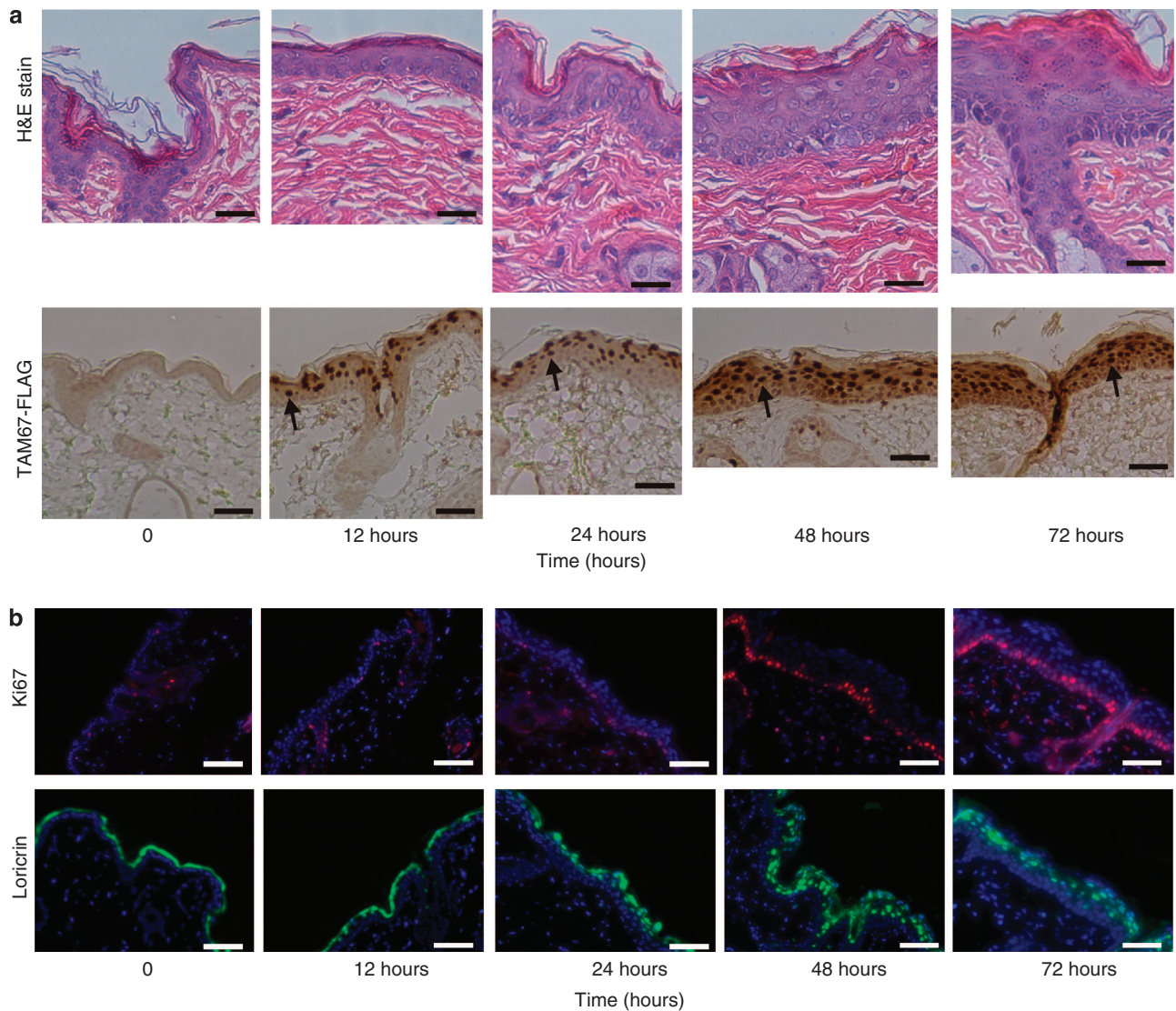
**Figure 2. Characterization of SKH1-TAM67-rTA mice.** SKH1-TAM67-rTA mice were administered drinking water supplemented with or not supplemented with  $2 \text{ mg ml}^{-1}$  doxycycline. (a) Doxycycline-treated and untreated littermates were photographed at 7, 14, and 21 days. (b,c) Enlarged images showing hyperkeratinization of the dorsal epidermis and foot in mice treated for 21 days with doxycycline. Panel b includes an image of an untreated littermate. (d) Histological appearance of the epidermis from an untreated littermate. The line labeled "E" marks the extent of the living epidermis. (e) Histological appearance of the epidermis from a 14 day doxycycline-treated littermate. The line labeled "E" marks the extent of the living epidermis. (f) Histological appearance of the cornified layer from e shows incomplete nuclear destruction (parakeratosis) in the cornified layer. The arrows indicate nuclei. (g,h) Loricrin distribution in untreated and 14 day doxycycline-treated SKH1-TAM67-rTA mice. The dotted lines indicate the dermal/epidermal junction. (i) Co-staining with 4' 6-diamidino-2-phenylindole (DAPI; nuclear) and anti-loricrin confirms loricrin nuclear distribution in the epidermis from a 14 day doxycycline-treated mouse. The scale bar =  $20 \mu\text{m}$ . (j) Immunoblot shows accumulation of TAM67-FLAG in a 14 day doxycycline-treated mouse and the absence of expression in an untreated littermate.

confirms that removal of doxycycline results in loss of TAM67-FLAG expression. Pseudoainhum is also observed on the digits. Figure 5f is an image showing development of edema in a digit undergoing pseudoainhum. This phenotype mimics the human disease (Seirafi *et al.*, 2011).

### Role of loricrin

An interesting and unexpected observation is the nuclear accumulation of wild-type loricrin in epidermal keratinocytes in TAM67-expressing mice (Figure 2i). This begs the question

of whether nuclear accumulation of wild-type loricrin is required for keratoderma phenotype development in this model. To assess this, we examined the impact of TAM67 expression in SKH1-TAM67-rTA mice that are homozygous, heterozygous, and null for loricrin. Mice were treated for 21 days with doxycycline prior to tissue harvest. Figure 6a confirms the TAM67 and loricrin expression status of the mice and shows that doxycycline treatment activates TAM67-FLAG expression at comparable levels in each strain. We next examined the impact of loricrin loss on the TAM67-dependent



**Figure 3. Time course of impact of TAM67-FLAG on epidermal end points.** SKH1-TAM67-rTA mice were administered  $2 \text{ mg ml}^{-1}$  doxycycline in drinking water, and at the indicated times tissue was harvested for characterization. (a) Epidermal morphology (hematoxylin and eosin (H&E) stain) and immunodetection of TAM67-FLAG using a peroxidase-based antibody. The arrows indicate nuclear TAM67-FLAG accumulation in cells of the suprabasal epidermis. (b) Immunodetection of Ki67 and loricrin. Ki67 is detected in the basal and immediately suprabasal cell layers in TAM67-FLAG-expressing mice. Loricrin shifts from staining the margin of the cornified layer at 0 and 12 hours to staining of nuclei at 24, 48, and 72 hours. Scale bars =  $50 \mu\text{m}$ .

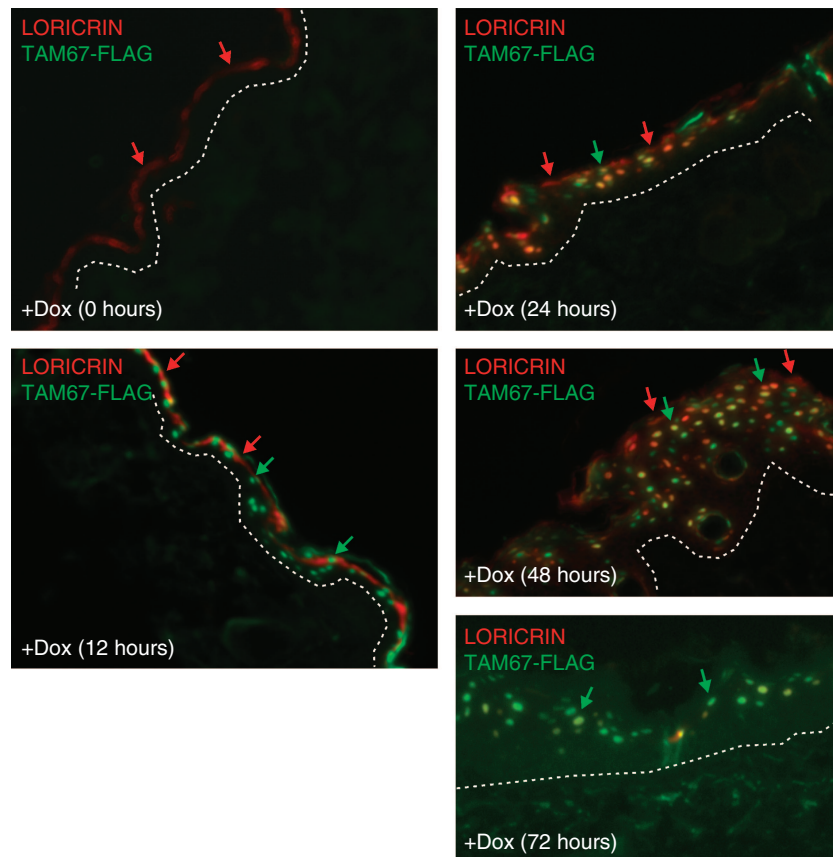
phenotype. Hematoxylin and eosin staining shows that TAM67 expression induces an epidermal hyperproliferative phenotype in the presence and absence of loricrin and that this is associated with activation of expression of the hyperproliferation-associated keratin, K6 (Figure 6b). As a control, we show that suprabasal expression of keratin 1 is maintained (Figure 6b). However, the most striking observation is that the epidermal scaling keratoderma-like phenotype develops unabated in mice with reduced or absent loricrin (Figure 6b and 6c).

## DISCUSSION

Keratodermas are a group of diseases characterized by thickening of the epidermis associated with marked hyperplasia and hyperkeratosis (Christiano, 1997). In the course of

studies designed to understand AP1 transcription factor function in epidermis, we produced mice in which AP1 transcription factor function is inactivated in the suprabasal epidermis (Rorke *et al.*, 2010). In this model, we induce suprabasal epidermal expression of TAM67, a dominant-negative form of c-jun that inhibits AP1 factor function, using a doxycycline-inducible system. TAM67 dimerizes with and inhibits transcriptional activation potential of all AP1 factors. We show that TAM67-FLAG interacts with AP1 response elements in a competitive manner and that this is associated with reduced expression of representative AP1 factor responsive genes. A remarkable finding is that these mice display a phenotype that includes all of the hallmarks of keratoderma. As AP1 factor inactivation in the basal epidermis





**Figure 4. Staining of loricrin (red) and TAM67-FLAG (green) showing that TAM67-FLAG appears in the nucleus at 12 hours and loricrin appears in the nucleus beginning at 24 hours.** Red arrows indicate loricrin localization, green arrows indicate TAM67-FLAG localization, and yellow signal indicates co-localization. TAM67-FLAG and loricrin co-localize in many but not all suprabasal nuclei.

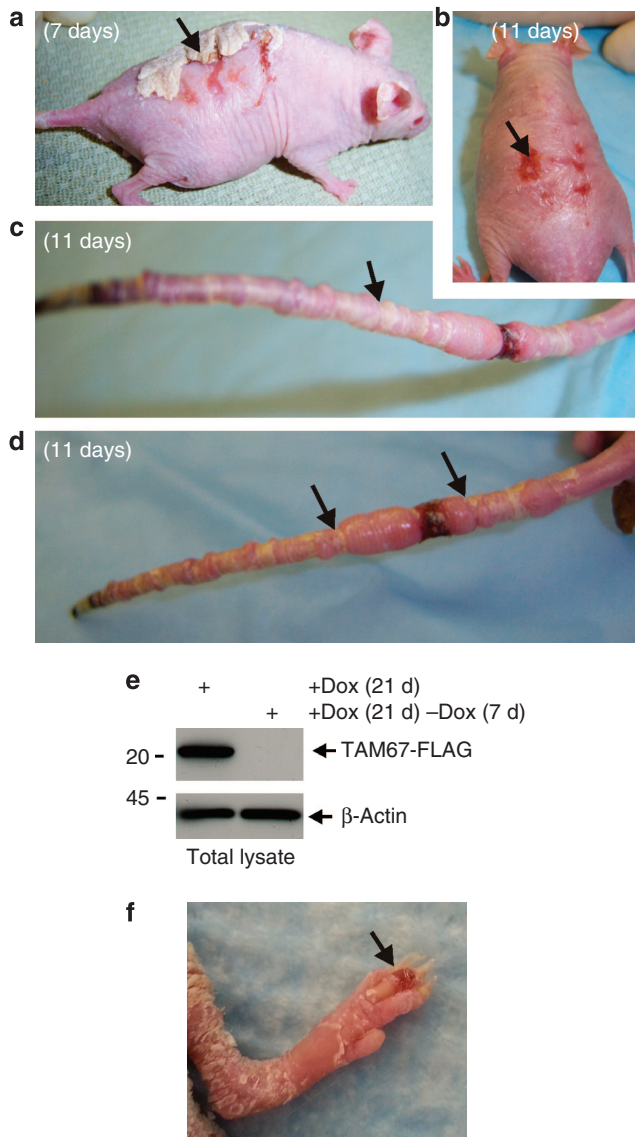
produces no such phenotype (Young *et al.*, 1999, 2002; Cooper *et al.*, 2003), this implies that epidermal compartment-specific changes in transcription factor signaling can cause keratoderma pathogenesis. Early changes include a progressive increase in skin thickness at 48 to 72 hours after TAM67 expression, and a parallel increase in cell proliferation. This is associated with nuclear accumulation of wild-type loricrin and changes in expression of differentiation markers. On a longer time scale, beginning after several days, the mice develop a honeycomb epidermal appearance. This is followed by accumulation of a thick layer of epidermal cornified material and tail and digit pseudoainhum and autoamputation. The fact that this phenotype so closely mimics human keratoderma strongly suggests that suppression of AP1 factor signaling is an important event in disease development.

Keratoderma includes both inherited and acquired forms. Inherited forms of this disease are associated with mutations in structural proteins (keratins), cornified envelope-associated proteins (loricrin, transglutaminase), cohesion proteins (desmoglein 1, plakophilin, desmoplakin), proteins of cell-to-cell communication (connexins), and transmembrane proteins (cathepsin C) (Christiano, 1997; Kimyai-Asadi *et al.*, 2002; Amagai and Stanley, 2012). Most of these mutations are autosomal dominant, although some are recessive (Kimiya-

Asadi *et al.*, 2002). The Vohwinkel syndrome and the Camisa variant of Vohwinkel syndrome are highly debilitating forms of the disease that display pseudoainhum and autoamputation of the digits and other extremities. Connexin mutations have been identified in Vohwinkel syndrome (Scott *et al.*, 2012), and targeted expression of mutant connexin in the suprabasal epidermis in mice produces a keratoderma-like phenotype (Schmuth *et al.*, 2004). A mutation in the loricrin gene, which leads to nuclear accumulation of loricrin, is associated with the Carmisa variant of Vohwinkel syndrome (Suga *et al.*, 2000). In addition, some forms of keratoderma are acquired in response to treatment with drugs, exposure to chemicals, malnutrition, infection, or response to cancer (Patel *et al.*, 2007). However, the underlying mechanisms that drive these diseases are not well understood. In particular, knowledge of changes in cell signaling is limited.

#### AP1 factors and keratoderma

AP1 transcription factors (c-jun, junB, junD, c-fos, Fra-1, and Fra-2) are important controllers of keratinocyte differentiation. They control differentiation and proliferation and are frequently perturbed in disease processes (Crish *et al.*, 1993; Welter *et al.*, 1995; Eckert *et al.*, 1997, 2004; Efimova *et al.*, 2003, 2004; Gerdes *et al.*, 2006). A role for transcription



**Figure 5. Pseudoainhum (autoamputation) in TAM67-expressing mice.** SKH1-TAM67-rTA mice were treated for 21 days with  $2 \text{ mg ml}^{-1}$  doxycycline, and the doxycycline was then removed for 7 or 11 days. (a,b) Appearance of mice at 7 and 11 days after discontinuation of doxycycline treatment. The accumulated cornified material is progressively shed from the skin surface. The arrows indicate the remaining cornified material and a residual scar. (c,d) Appearance of the tails of two mice after 21 day doxycycline treatment followed by 11 days without doxycycline. The arrows show representative pseudoainhum bands on each tail. In some mice the tail is lost because of autoamputation. (e) An immunoblot showing TAM67-FLAG level in 21 day doxycycline treatment mice versus mice treated for 21 days followed by 7 days of discontinued treatment. (f) Early-stage pseudoainhum of digits in mouse treated for 21 days with doxycycline. The arrow indicates pseudoainhum-associated edema.

factors in the development of keratoderma has not been studied. The present study shows that direct suppression of AP1 transcription factor function can produce a keratoderma phenotype. It is interesting that the phenotype is observed in the absence of mutation in proteins known to be mutated in

keratoderma, and that the phenotype is only observed when AP1 factor function is selectively inactivated in the suprabasal epidermis. Suprabasal AP1 factor inactivation appears to inhibit/delay normal keratinocyte differentiation and also cause an increase in basal layer proliferation. The latter suggests a potential indirect effect due to incomplete differentiation and may involve paracrine mediators. Given the central role that AP1 factors have in regulating keratinocyte function, it is perhaps not surprising that reducing AP1 factor signaling produces a keratoderma phenotype. It is interesting to speculate that suppressed AP1 factor function may be an underlying response that is common in a range of keratoderma types—both genetically inherited and acquired.

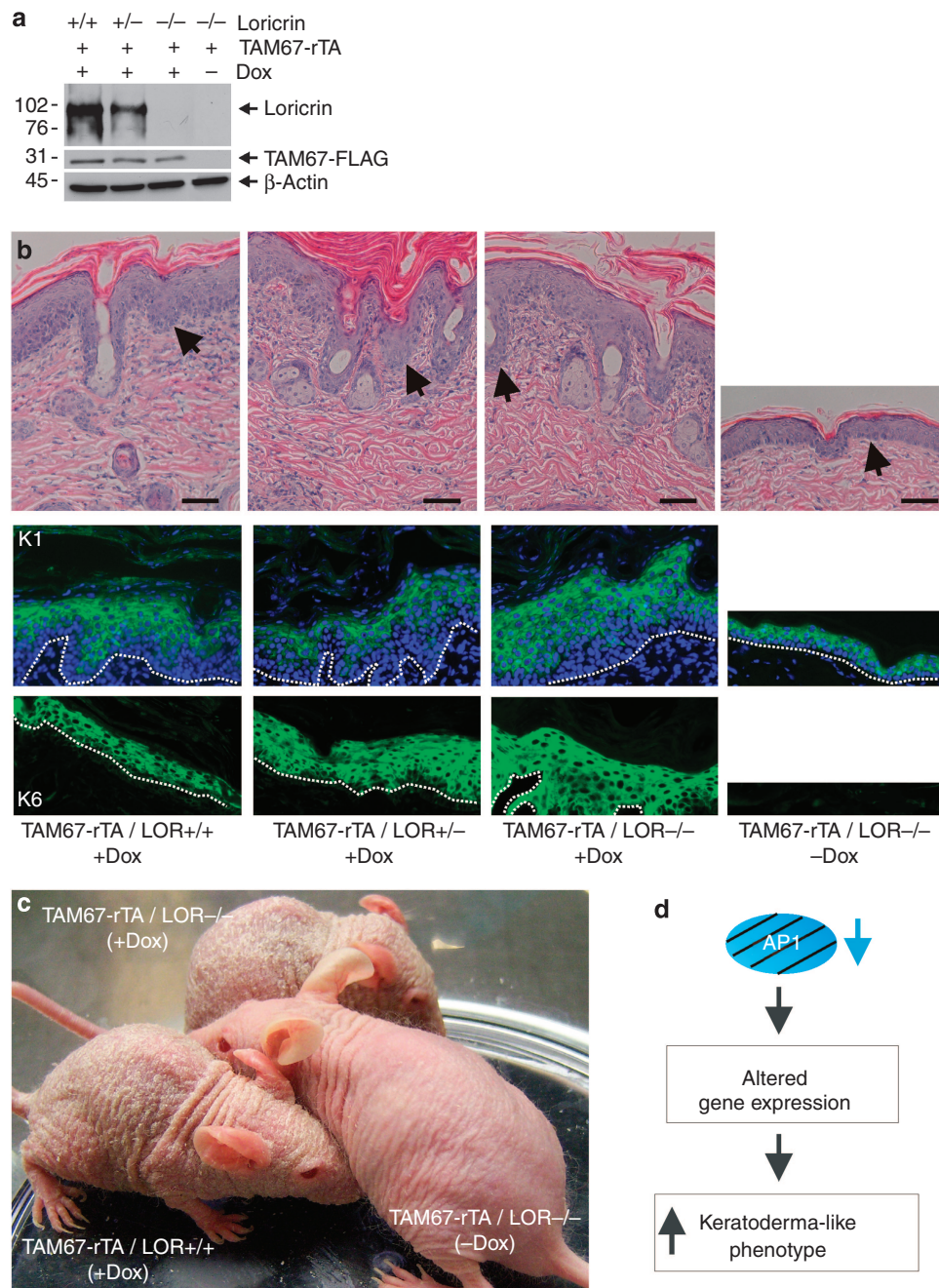
### Role of loricrin

An interesting property of the mice produced in the present study is the presence of two signature features commonly associated with the Camisa variant of Vohwinkel syndrome (loricrin keratoderma). These are nuclear loricrin accumulation and pseudoainhum/autoamputation. Loricrin keratoderma is an interesting form of the disease that is characterized by honeycomb-like palmoplantar keratoderma, hyperkeratosis, tissue buildup on the dorsal hands and feet, nuclear accumulation of mutant loricrin, and pseudoainhum and autoamputation of extremities. Loricrin is a structural protein that is a component of the cornified envelope (Mehrel *et al.*, 1990; Candi *et al.*, 2005). In normal epidermis, it is produced during the terminal stages of differentiation and is then crosslinked as part of the cornified envelope (Mehrel *et al.*, 1990; Hohl *et al.*, 1991). In keratoderma, mutant loricrin accumulates in the nucleus due to a frameshift mutation that adds a nuclear localization signal at the c-terminal end of the protein (Maestrini *et al.*, 1996; Korge *et al.*, 1997; Takahashi *et al.*, 1999; Matsumoto *et al.*, 2001; O'Driscoll *et al.*, 2002; Gedicke *et al.*, 2006; Drera *et al.*, 2008). However, what loricrin does in the nucleus is not known. Although wild-type loricrin is not thought to cause disease, our observation of its accumulation in the nucleus at early times (12 to 24 hours) during phenotype development suggests that it could be driving the phenotype. Moreover, some investigators have described nuclear localization of wild-type loricrin (Ishida-Yamamoto *et al.*, 1993; Yoneda *et al.*, 2010a, b). We do not know why wild-type loricrin accumulates in the nucleus in the presence of TAM67, but we presume that unknown factors are produced that facilitate this movement. To determine whether wild-type loricrin has a role in disease development, we studied phenotype development in a loricrin-null background. These studies clearly indicate that a complete phenotype develops in the absence of loricrin, indicating that wild-type loricrin has no role. Thus, suprabasal AP1 factor inactivation is sufficient to drive the phenotype.

### Pseudoainhum

Pseudoainhum and autoamputation is associated with the Vohwinkel syndrome and the Camisa Vohwinkel syndrome variant (Camisa and Rossana, 1984; Korge *et al.*, 1997; Maestrini *et al.*, 1999; Schmuth *et al.*, 2004). In our model,





**Figure 6. Suppression of suprabasal AP1 factor signaling is sufficient for phenotype development; -loricrin is not required.** (a) Immunoblot confirmation of mouse genotype. SKH1 mice of the indicated TAM67-FLAG and loricrin genotypes were treated for 21 days with (+) or without (-) 2 mg ml<sup>-1</sup> doxycycline. At 21 days epidermal extracts were prepared for detection of the indicated proteins. (b) Hematoxylin and eosin, K1, and K6 staining of epidermis from 21 day doxycycline-treated mice. Arrows or dotted lines indicate the epidermal/dermal junction. Scale bars = 50 μm. (c) Appearance of mice of the indicated genotype after 21 days of doxycycline treatment. Absence of loricrin did not prevent phenotype development. (d) Hypothesis: inhibition of suprabasal AP1 factor signaling produces a keratoderma-like phenotype that closely resembles human keratoderma by altering gene expression.

suprabasal AP1 factor inactivation produces digit and tail pseudoainhum and autoamputation. The phenotype is particularly evident on the tail. This is a complex aspect of keratoderma that is not well understood (Rashid *et al.*, 2007). The disease is thought to involve a complex series of events that include abnormal vascular ligation, secondary atrophy and fibrosis, and constriction leading to autoamputation

(Rashid *et al.*, 2007). It also may involve genetic or systemic components, as the response is often symmetric and bilateral (Rashid *et al.*, 2007). It is particularly interesting that compromised suprabasal AP1 factor function may trigger a series of events that drive this phenomenon.

The fact that the phenotype is inducible and reversible in our model suggests that it will be possible to study



pseudoainhum and autoamputation development and regression. On the basis of these findings, we propose that reduced AP1 transcription factor function in the suprabasal epidermis leads to presently unknown changes in gene expression that drives the keratoderma phenotype (Figure 6d). We further propose that changes in AP1 factor signaling may be a common event in multiple keratoderma types and may be involved in the pathogenesis of acquired keratoderma that develops in response to environmental factors.

## MATERIALS AND METHODS

### SKH1-TAM67-rTA mice

TAM67 is a dominant-negative form of c-jun that lacks the c-jun amino terminus (Brown *et al.*, 1994). TAM67 forms complexes with all jun and fos transcription factors, but when these complexes interact with DNA response elements they fail to activate transcription (Brown *et al.*, 1994). Thus, it can be used to create an AP1 factor function-deficient environment. We cloned TAM67-FLAG into pTRE-Tight to produce pTRE-Tight-TAM67-FLAG. The TetO-TAM67-FLAG-SV40 transcription cassette from this plasmid was microinjected into B6SJL embryos (Rorke *et al.*, 2010) to produce TetO-TAM67-FLAG<sup>TCG/-</sup> (strain 44) mice. A FLAG epitope was included at the carboxyl terminus of TAM67 to facilitate TAM67 detection. These mice were mated with FVB hINV-rTA<sup>TCG/-</sup> mice (Jaubert *et al.*, 2004) to produce bi-transgenic B6SJL/FVB TAM67-FLAG<sup>TCG/-</sup>/hINV-rTA<sup>TCG/-</sup> mice (TAM67-rTA) (Rorke *et al.*, 2010).

In the present studies, we transferred the TetO-TAM67-FLAG and hINV-rTA genes to the SKH1 genetic background by breeding for five generations. These mice were then bred to produce SKH1-TAM67-rTA mice. Treatment of these mice with doxycycline results in involucrin promoter-specific targeting of rTA to these layers (Rorke *et al.*, 2010). The mice were genotyped using DNA-dependent PCR and primers that detect each transgene (Rorke *et al.*, 2010). The SKH1 genetic background was utilized in these studies because the mice are hairless, and this facilitates visualization of the epidermis. SKH1 mice are immune competent. Loricrin knockout mice (Koch *et al.*, 2000) were bred for five generations into the SKH1 background. The wild-type and loricrin knockout genotypes were detected using 5'-CACTCCCAGCACAATAAGAA-3' and 5'-TTGCATCGCATGTCT GAGT-3', respectively, as the forward primers. The reverse primer was 5'-GCACTGGGAGAGTGGTAAGC-3' for both wild-type and knockout mice. The primers produce bands at ~350 (knockout) and 650 (wild type) nucleotides. The mice were housed in the University of Maryland School of Medicine animal facility in compliance with the NIH regulations.

### Antibodies and immunological methods

Immunofluorescence was performed using paraffin-embedded formalin-fixed sections as previously described (Crish *et al.*, 1998, 2002, 2006). Loricrin (PRB-145P), K1 and K6 antibodies were from Covance (Emeryville, CA). Ki67 (TEC-3) antibody was from Dako (Cupertino, CA), and  $\beta$ -actin (A5441) and FLAG (M2) (F4049)-specific antibodies were obtained from Sigma (St Louis, MO). Primary antibody localization was visualized using an appropriate fluorophore-conjugated secondary antibody. For immunoblot, the epidermis was separated from the dermis, frozen in liquid nitrogen, pulverized, and then suspended in dye-free Laemmli sample buffer. The suspension

was sonicated, particulates were removed by centrifugation, and soluble extract was electrophoresed on a polyacrylamide gel and transferred to nitrocellulose for immunoblot (Crish *et al.*, 1998, 2002, 2006). Unless otherwise indicated, the experiments were repeated three separate times, and sections and extracts were monitored from the epidermis of three mice per treatment group.

### Gel mobility shift assay

Epidermis was isolated for preparation of nuclear extract using NE-PER Nuclear and Cytoplasmic Extraction Reagent (78833, Pierce Biotechnology, Rockford, IL). TAM67-FLAG binding to double-stranded AP1 consensus (AP1c) oligonucleotide 5'-CGCTTGAT-GAGTCAGCCGGAA-3' (E320A, Promega, Madison, WI, AP1 site in bold) was monitored by gel mobility shift assay. Three micrograms of nuclear extract were incubated for 30 minutes at room temperature in a volume of 20  $\mu$ l containing 20 mM HEPES, pH 7.5, 10% glycerol, 50 mM KCl, 2 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 0.5 mM DTT, 1  $\mu$ g ml<sup>-1</sup> poly(dI:dC), 0.1 mg ml<sup>-1</sup> bovine serum albumin, and 40,000 c.p.m. radioactive double-stranded AP1c-P<sup>32</sup> or AP1-5-P<sup>32</sup> oligonucleotide. For competition studies, a fixed (40-fold) molar excess of nonradioactive AP1c competitor oligonucleotide was added to the DNA binding reaction. For the gel mobility supershift assay, 2  $\mu$ g of antibody specific for FLAG (F3165, Sigma) was added to the reaction mixture and incubated for 1 hour at 25 °C. The <sup>32</sup>P-labeled probe was then added, and the incubation was continued for an additional 30 minutes at 25 °C. Protein-DNA complexes were resolved in 6% polyacrylamide gels under nondenaturing conditions (Adhikary *et al.*, 2004, 2005).

### CONFLICT OF INTEREST

The authors state no conflict of interest.

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